Chapter 13

Regulation of *Aspergillus flavus* aflatoxin biosynthesis and development

Jeffrey W. Cary¹, Leanne Szerszen², and Ana M. Calvo²

¹ARS, U.S. Department of Agriculture, Southern Regional Research Center, New Orleans, LA 70124
²Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115

The filamentous fungus *Aspergillus flavus* produces a family of potent mutagenic and carcinogenic polyketide-derived compounds collectively known as aflatoxins. These secondary metabolites contaminate a number of oilseed crops during growth of the fungus and this can result in severe negative economic and health impacts. The biosynthesis and regulation of these toxins represent one of most studied areas of all the fungal secondary metabolites. Much of the information obtained on the AF biosynthetic genes and regulation of AF biosynthesis was obtained through studies using *A. flavus* and *A. parasiticus* and also the model fungus *Aspergillus nidulans* that produces sterigmatocystin (ST), the penultimate precursor to AF. There has long been anecdotal evidence of a genetic linkage between production of secondary metabolites and fungal morphogenesis however the exact mechanism of this relationship was not clear. A breakthrough in the genetic mechanisms governing AF production and *A. flavus* development was made upon the discovery in *A. nidulans* of a G-protein-mediated signaling pathway that regulated both ST biosynthesis and asexual conidiation. Further studies in *A. nidulans* and *A. flavus* and also of the fungus-host plant interaction have identified a number of genetic factors that link secondary metabolism and morphological differentiation processes in *A. flavus* as well as filamentous fungi in general. The focus of this review is to provide an overview of research that characterized the genes involved in the biosynthesis and
regulation of AF in *A. flavus*, how environmental and nutritional factors control expression of these genes, and the role of global regulators in AF production, fungal development and virulence. The impact of fungal whole genome sequence and microarray technology in the identification of novel genes involved in the regulation of AF production and development as well as virulence are also discussed.

The Aflatoxin Biosynthetic Gene Cluster

Linkage of AF pathway genes was first evidenced in an *A. parasiticus* cosmid clone that harbored both the *aflD (=nor-I)* and *aflM (=ver-I)* genes (1) and soon thereafter transcriptional mapping of overlapping cosmid clones of *A. parasiticus* and *A. flavus* established that the genes involved in AF biosynthesis were present on a single gene cluster (2, 3). Four structurally related forms of AFs occur in nature depending on the presence of the dihydro-bisfurans rings (B) and G1 or the tetrahydro-bisfurans rings (B2 and G2). In general, *A. parasiticus* produces both B and G AFs while *A. flavus* only produces the B forms. In *A. parasiticus* and *A. flavus* the enzymes and regulatory proteins required for AF production are encoded by at least 29 genes that are clustered in a 70-kb DNA region. *Aspergillus nidulans* has a similar biosynthetic cluster required for the production of the mycotoxin ST, an intermediate in AF formation, that spans about 60 kb and contains homologs of many of the AF biosynthetic genes (4). Both pathways contain a gene that encodes a positive-acting, pathway-specific transcriptional regulator, AflR, that is responsible for the co-regulation of many if not all of the AF/ST biosynthetic genes (5, 6).

Ehrlich and collaborators (7, 8) sequenced and compared the AF gene clusters from a number of *Aspergillus* species including *A. nomius*, two sclerotial morphotypes of *A. flavus*, and an unnamed *Aspergillus* species from West Africa that produces both B and G AFs. They found that the gene order (Fig. 1) for all of the isolates was the same as that for the *A. parasiticus* AF cluster. Twenty two of these genes were homologous to genes in the *A. nidulans* ST cluster, however the order and direction of transcription of some of the ST genes differed from that of the AF gene cluster. A comparison of the AF and ST gene clusters is provided by Ehrlich et al. (7). Clusters varied in length from 66.1 kb for the small (S) sclerotial morphotype and 66.5 kb for the large (L) morphotype of *A. flavus*. Sclerotia are survival structures that remain dormant in adverse environments and then germinate under favorable conditions. The difference in cluster size between the sclerotial morphotypes is due in large part to deletions of portions of the coding and intergenic regions of the *aflF (=norB)* and *aflU (=cypA)* genes. Loss of *aflU* function results in loss of the ability to produce G AFs. Deletions are not only limited to the *aflF-aflU* gene region. An examination of 38 nonaflatoxigenic *A. flavus* field isolates showed 8 distinct patterns of large (≥40 kb) deletions involving the AF gene cluster some extending from within the cluster to the end of the chromosome (9). The *A. nomius* cluster was found to be
68.4 kb in length. In all the AF gene clusters analyzed, the \textit{aflF} gene represented the proximal end of the cluster while, \textit{aflY} (=hypA), a gene of unknown function was at the distal end. New evidence suggests that the \textit{nadA} gene, adjacent to \textit{aflY} and once believed to be part of a sugar utilization gene cluster, is actually involved in biosynthesis of G AFs and therefore constitutes the distal end of the cluster (10). Of the 29 genes identified in the pathway, only 7 (\textit{aflF}, \textit{aflT}, \textit{hypB1}, \textit{aflE} (=norA), \textit{hypE}, \textit{hypB2}, and \textit{aflY}) have yet to have the function of their protein product determined experimentally.

Other \textit{Aspergillus} section \textit{Flavi} species such as the nonaflatoxigenic \textit{A. oryzae} and \textit{A. sojae} are generally recognized as safe (GRAS) and are used in food fermentations. Both fungi harbor an AF gene cluster however specific defects and/or deletions in the \textit{afiR} gene and other AF pathway structural genes result in lack of AF production (11-14). Both the AF clusters of \textit{A. flavus/parasiticus} and \textit{A. oryzae} and the ST cluster of \textit{A. nidulans} are located in the subtelomeric regions of their respective chromosomes (15). In general, there appears to be a preference for the location of secondary metabolic gene clusters to the subtelomeric regions. Subtelomeric regions are known to be active regions for intra-molecular recombinations, insertions or deletions, and translocations (15). Therefore, the proximity of the AF cluster to the telomere may facilitate the rapid reorganization and evolution of these genes in a species-specific fashion. Carbone et al. (15) have identified specific gene modules that exist in the AF and ST clusters. They postulate that these modules have arisen from gene duplications that retain the pre-duplicated gene’s function or the duplicated copy’s function may augment a specific pathway function. Additionally, the duplicated copy may evolve a completely new function. They hypothesized that it is possible that genes may become separated after their duplication and that differences in gene order between AF and ST clusters may be the result of gene reorganization in an ST-producing ancestor.
Figure 1. (A) Metabolic precursors of aflatoxin: NOR, norsolorinic acid; AVN, averantin; HAVN, hydroxyaverantin; AVNN, averufin; AVF, averufin; HVN, hydroxyversicolorone; VHA, versicolorone hemiacetal acetate; VERB, versicolorin B; VERA, versicolorin A; ST, sterigmatocystin; OMST, O-methylsterigmatocystin; AF, aflatoxin. Schematic representation of the (B) aflatoxin and (C) sterigmatocystin biosynthetic gene clusters. The direction of transcription is indicated by arrows. In the AF cluster the gene designations above the schematic represent the afl nomenclature while those below represent the enzymatic function nomenclature. In the ST cluster the gene designations above represent the stc nomenclature and those below indicate the AF cluster homolog.
Transcriptional Regulation of Aflatoxin Biosynthesis and Development

Aflatoxin Pathway-Specific Regulators

A positive regulatory gene, aflR, encodes a sequence-specific, Gal4-type C6-zinc binuclear cluster DNA binding protein that is required for transcriptional activation of the AF structural genes (16, 17). AfiR also regulates the expression of A. nidulans ST biosynthetic genes (5). The AflR protein binds to the palindromic sequence 5'-TCGN3CGR-3' within the promoter region of AF structural genes (5, 6). Interestingly, a study by Price et al. (18) using A. flavus EST microarray transcription profiling studies identified two additional genes (niiA and hlyC) located well outside of the AF gene cluster that were upregulated under AF-conducive conditions. In these genes, the consensus AflR binding sites were observed, however, they were located from 1.8 to 2.3 kb upstream of the translational start sites. A number of studies have also shown that elements upstream of the aflR coding region may serve as binding sites for proteins that negatively regulate aflR expression (19-21). In addition aflR influences A. flavus morphogenesis. Loss of aflR does not result in loss of spore or sclerotial formation, however, it does impact the numbers of spores or sclerotia being formed (22).

Another gene that appears to play a role in the regulation of AF production is aflS (=aflJ). This gene is adjacent to and divergently transcribed from aflR. AflS does not demonstrate any significant homology to other genes or deduced protein sequences present in the genome databases (23). Though the exact function of AflS has not been determined, it is required for production of wild-type levels of AF and it has been shown to interact with AflR (23-25). Interestingly, though aflS overexpression increased AF production its overexpression did not result in elevated transcription of mid- to late-AF pathway intermediate genes such as aflM (=ver-1) or aflP (=omtA). However, transformants expressing aflR and aflS produced five times more aflC (=pksA) transcripts and four times more aflD (=nor-1) transcripts than strains expressing only aflR (25). This would suggest that aflS modulates the regulation of early genes in the AF biosynthetic pathway.

Global Regulatory Factors

Early studies by Kale et al. (26) suggested a genetic connection between AF production and development in A. parasiticus. This observation was extended by the characterization of a G-protein-mediated signal transduction pathway in the model fungus A. nidulans that regulates both asexual conidiation and ST production (27). This topic has been covered extensively in previous reviews (28-30). In general, G-protein-coupled receptors activated by a ligand transmit the signal to two downstream signaling pathways: a G-protein-cAMP-dependent kinase (28) and/or mitogen-activated protein kinase pathway, affecting several cell functions, including morphogenesis and mycotoxin biosynthesis (31, 32).
VeA

The veA gene is well known as a regulator of light-dependent morphogenesis in aspergilli. *A. nidulans* cultures exposed to light develop asexually forming abundant air-borne conidia, while in the dark sexual development is favored resulting in the formation of fruiting bodies called cleistothecia (33). Although the veA gene product does not present homology with any other proteins of known function, veA has been found in the genome of many filamentous fungi, being particularly conserved in Ascomycetes (Calvo et al., unpublished). Deletion of veA blocks cleistothecia production (34). It is also known that the veA gene plays a global regulatory role in the synthesis of secondary metabolites, including mycotoxins (34-38). Studies have demonstrated that veA controls the transcription of genes necessary for the synthesis of AF in *A. flavus* and *A. parasiticus* and ST in *A. nidulans*. VeA was shown to be required for the expression of aflR and subsequent activation of AF/ST biosynthetic genes (35-38). Importantly, the *A. flavus* and *A. parasiticus* deletion mutants were also unable to produce sclerotia, further supporting the hypothesis that sclerotia could be sterile vestiges of cleistothecia (Fig. 2) (35, 36, 39).

![Figure 2](image_url)

**Figure. 2. Analysis of *A. flavus* veA mutant (ΔveA). Left panel: TLC analysis of wild-type (WT) and ΔveA strains showing lack of AF production in the mutant. Right panel: YGT agar cultures of WT and ΔveA strains following 5 d growth. Note abundant sclerotia (dark structures) in WT and absence in ΔveA.**

These findings on the role of VeA in the regulation of secondary metabolism and morphogenesis, together with the fact that veA has only been found in fungi (phylogenetic studies in Calvo’s lab, unpublished), suggest that veA or veA-dependent genes could serve as targets for development of strategies to decrease the detrimental effects of mycotoxin contamination in our food supplies (40, 41). The veA gene also regulates the synthesis of additional secondary metabolites such as cyclopiazonic acid and aflatrem toxins in *A. flavus* and the antibiotic penicillin in *A. nidulans* that were reduced or veA deletion mutants (34, 36).

As mentioned above, VeA function is light-dependent. Interestingly, Stinnett et al. (42) showed that *A. nidulans* VeA migration to the nucleus is
light-dependent. While in the dark VeA is located mainly in the nuclei, under light VeA is found abundantly in the cytoplasm. Blue light (440–500 nm) had a similar effect to that observed with white light, preventing an efficient accumulation of VeA in the nuclei (42). The effect of exposure to red light (625–740 nm) was similar but more moderate in comparison with blue and white light. Blumenstein et al. (43) reported that a phytochrome-like protein called FphA acts as a red-light sensor in A. nidulans and represses sexual development and ST production. Previous studies suggested a possible interaction of VeA with light-responsive proteins (42, 44) and it was recently demonstrated that FphA protein interacts with VeA as part of a protein complex in the nucleus (45). These studies indicated that red and blue-light perception occurs in an integrative way in the nuclear VeA-protein complex affecting morphogenesis and secondary metabolism. Furthermore, VeA abundancy in the nucleus was negatively affected by FphA in the light. This, together with the fact that nuclear concentration of VeA is also reduced by blue light (42) provide further evidence that both, red- and blue-light sensing systems are influencing VeA nuclear localization and consequently toxin production. The fact that the VeA complex includes proteins that respond to external stimuli, such as light, and proteins that affect the expression of secondary metabolic gene clusters, such as LaeA, further elaborated below, suggested a possible scaffold role for VeA. A scaffold-like role for VeA in the nucleus has been identified. In A. nidulans nuclei, VeA was also shown to physically interact with VeIB (a velvet-like protein), which is expressed during sexual development (46). VeA is believed to bridge VeIB to LaeA. Deletion of either velB or veA resulted in defects in both ST and cleistothecial production.

Our lab has demonstrated that VeA also plays a role in A. flavus virulence (Duran, Cary and Calvo, submitted). Virulence of A. flavus on peanut and maize seeds was reduced in the absence of the veA gene product. Generation of air-borne asexual conidia was reduced in viable or non-viable peanut seeds and in viable maize seed. Production of AF and sclerotia in peanut and maize seed was completely blocked when infected with the A. flavus veA mutant (ΔveA). In planta inoculated cotton bolls examined 3 weeks post-inoculation (soon after boll opening) showed that conidiation was decreased in bolls inoculated with the ΔveA strain and spread [as determined by intercarpellary membrane damage (47)] of the ΔveA strain to locules adjacent to the inoculated locule was less than observed with the wild-type veA strain (data not shown, Duran, Cary and Calvo, submitted). As observed in peanuts and maize, no AF was produced in seed harvested from cotton bolls that had been inoculated with the ΔveA strain while AF was present in seed from wild-type veA inoculated bolls (data not shown, Duran, Cary and Calvo, submitted).

LaeA

The nuclear regulator LaeA has been shown to govern production of multiple secondary metabolites in Aspergillus species (48-51). LaeA is a positive regulator of aflR expression, which in turn activates the expression of AF/ST genes. It was shown to be a negative regulator of veA expression in A.
flavus and laeA deletion mutants demonstrated decreased AF production and no sclerotia were formed (48). This would suggest that both veA and laeA are required for AF/ST production and sclerotial formation through the formation of a regulatory protein complex in the nucleus and that regulation of veA by laeA may represent an internal mechanism to balance stoichiometry of this complex (48). As observed with A. flavus veA mutants, A. flavus laeA mutants also were less able to colonize peanut and maize seed in vitro. Interestingly, an A. nidulans laeA mutant was preferentially attacked by the fungivorous springtail Folsomia candida indicating that the arthropod could sense the presence of a secondary metabolite(s) being produced by the wild-type fungus (52).

The LaeA deduced amino acid sequence indicated that this protein can function as a methyltransferase, as it harbors a S-adenosyl methionine binding domain (53). ST cluster expression analysis showed that regulation by laeA is spatially limited to the cluster genes, not affecting genes adjacent to the cluster (50, 53-55). This was also found to be the case for the A. nidulans terrequinone A cluster (54). Based on preliminary evidence, it is hypothesized that LaeA may influence chromatin structure at cluster loci thus controlling transcriptional activation of cluster genes (55, 56).

The Effect of Histone Acetylation on Toxin Biosynthesis

AF biosynthesis is influenced by a number of nutritional and environmental factors (see below). Stimulation of AF biosynthesis by high levels of glucose and cAMP has been demonstrated and explained, at least in part, by production of elevated levels of AflR that in turn increases transcription of AF biosynthetic genes. However, studies have suggested that other proteins are needed to assist in the binding of AflR to the promoters of AF biosynthetic genes and also for optimal transcriptional activity. Roze et al. (57) identified a binding site (CRE1), unique from the AflR binding site (AflR1), in the promoter of the aflD gene, which served as a cAMP-response element. Mutation in CRE1 or AflR1 caused up to a three-fold decrease in cAMP-mediated stimulation of aflD promoter activity. They demonstrated that the CRE1 site was required for binding of a 32 kDa protein (CRE1bp) and hypothesized that CRE1bp interacts with AflR to assist in its binding to the aflD promoter. Several CRE1-like binding sites are found in different AF gene promoters, so this interaction may extend beyond that of just the aflD gene.

Importantly, CRE1 has been shown to recruit histone acetyltransferase (HAT) to promoter regions, leading to acetylation of histones, in particular histone H4, which has been demonstrated to enhance transcriptional activation (58-60). In a recent study, Roze et al. (61) demonstrated a positive correlation between the initiation and spread of histone H4 acetylation in AF gene promoters, leading to the establishment of AF pathway gene expression and AF accumulation. AF gene transcription is enhanced by AflR-binding to the promoter regions as access is made available by changes in chromatin conformation. Previous studies have shown that when AF gene promoter-reporter fusions were integrated outside of the AF cluster, promoter activity was greatly decreased supporting the model of specific regulation of cluster
expression (62). The role of epigenetic regulation of secondary metabolic gene clusters was further supported by recent evidence that deletion of the histone deacetylase (HDAC) gene, \textit{hdaA}, in \textit{A. nidulans} resulted in activation of transcription of genes of the ST and penicillin biosynthetic gene clusters (63). Interestingly, while they showed that ST and PN production could be restored in a \(\Delta\text{laeA} \Delta\text{hdaA}\) double mutant, levels of these metabolites were not as high as in the \(\Delta\text{hdaA}\) alone. This suggested that LaeA and HdaA operate through different mechanisms.

**Effect of Environmental Factors on Aflatoxin Production and Fungal Development**

**Role of Nitrogen, Carbon, and pH**

Production of AF is also under the control of a number of global regulatory networks that respond to environmental and nutritional cues. These include responses to nutritional factors such as carbon and nitrogen sources and environmental factors such as pH, light, oxidative stress and temperature. Globally-acting regulatory proteins such as AreA involved in nitrogen signaling, CreA involved in carbon signaling, and PacC involved in pH mediated signaling either positively or negatively influence AF production.

In general, nitrate inhibits AF production while ammonium salts are conducive (64). However, nitrate has been shown to enhance ST production in \textit{A. nidulans} while ammonium-based media repressed ST (65). Nitrogen metabolism in fungi is regulated by the globally-acting transcription factor AreA (66). Over-expression of the \textit{afiR} gene in \textit{A. parasiticus} resulted in release of nitrate inhibition on AF biosynthesis indicating that AreA imparts its control on toxin synthesis either directly or indirectly via \textit{afiR} (67). Electrophoretic mobility shift assays (EMSAs) indicated that the \textit{A. parasiticus} AreA binds within the \textit{afiR-afiS} intergenic region and a number of putative AreA GATA binding sites are present within this region (21). Analysis of the effects of nitrate on AF production and expression of \textit{afiR} and \textit{afiS} in a number of \textit{A. flavus} strains indicated variability in nitrogen regulation and this variability could often be found to correspond to differences in the number of GATA sites near the \textit{afiS} tsp (68). In addition, nitrogen source can also influence formation of sclerotia in \textit{A. flavus}. Studies of \textit{A. flavus} growth on agar media containing either nitrate or ammonium as the sole nitrogen source showed that sclerotial development occurred with nitrate but not with ammonium [see Genomics section and (69)].

AF biosynthesis is induced by simple sugars such as glucose and sucrose that are either present or generated by fungal hydrolytic enzymes during invasion of seed tissues (70, 71). When molasses was added to three commonly used growth media, conidial production was stimulated while AF production was reduced in a small sclerotial \textit{A. flavus} isolate whereas sclerotial formation increased or decreased depending on the medium (72). There is no evidence for the involvement of carbon catabolite repression by CreA in regulation of AF
production as in most cases glucose stimulates AF production. No putative CreA binding sites have been identified in the promoters of AF pathway genes. A sugar utilization gene cluster has been identified distal to the *nadA* gene of the AF cluster in *A. flavus* as well as a number of other *Aspergillus* section *Flavi* species but it does not flank the ST cluster of *A. nidulans* (7, 73). Though not experimentally proven to be linked to AF production, the fact that this cluster is conserved among section *Flavi* species suggests that a higher-order chromatin structure encompassing both the AF and sugar cluster could be important for expression of genes in the clusters (7).

AF and ST production, in general, is greatest in acidic medium and tends to decrease as the pH of the medium increases (74). An atypical, West African strain of *A. flavus* was identified that produced less AF in acidic medium (75). The strain is designated *A. flavus* SBG as it produced both AFB1 and AFG1 whereas most *A. flavus* strains only produce the B AFs. Interestingly, the changes in AF production between the SB and SBG strains did not correlate well with changes in *afiR* expression indicating that pH may be exerting its effects on other cellular metabolic processes that in turn regulate AF biosynthesis. Response to changes in pH is regulated by the globally-acting transcription factor PacC that is post-translationally modified by a pH-sensing protease (76). A number of putative PacC binding sites have been identified in the promoters of AF biosynthetic genes and could be involved in negatively regulating AF biosynthesis during growth at alkaline pH (6, 77). Fungal development also appears to respond to changes in pH as sclerotial production was found to be reduced by 50% at pH 4.0 or less while AF production was at its maximal (78).

**Role of Plant Metabolites**

A number of plant-based metabolites have been shown to reduce AF production as well as alter fungal development (79). These include volatile aldehydes (80-82), flavanoid compounds (83), neem leaf extracts and jasmonic acid (84, 85). Volatile aldehydes, jasmonic acid, and methyl jasmonate are all biologically-active end products of the LOX pathways in plants. LOX pathway metabolic precursors (i.e. oxylipins) have been shown have significant effects on fungal development and toxin production (see below) and these effects are more than likely mediated by the above described end products of the LOX pathways that can function as signaling molecules in transduction pathways that regulate a number of biological processes (86).

Ethylene and CO2 treatment have been shown to reduce AF production in *A. parasiticus* (87, 88). Treatment with ethylene reduced AF accumulation in a dose-dependent manner with a 10-fold reduction observed when *A. parasiticus* was treated with 146 ppm ethylene. CO2 at 0.1% also reduced AF accumulation about 5-fold however adding more CO2 (0.7 or 3.0%) reversed the inhibitory effect. Treatment of infected peanut seeds with differing concentrations of ethylene and/or CO2 inhibited AF accumulation up to 5-fold. Other volatile compounds described to affect AF production are 2-ethyl-1-hexanol, and 2-buten-1-ol. 2-ethyl-1-hexanol stimulates AF production in *A. parasiticus* (89). However, 2-buten-1-ol showed a dose-dependent up-regulatory or down-
regulatory effect not only on AF gene transcription and AF accumulation, but also on production of asexual spores. Both 2-ethyl-1-hexanol and 2-buten-1-ol were found to be produced by *A. parasiticus* and at a higher level by *A. nidulans*. Molyneux et al. (90) showed that AF production is markedly decreased by the presence of natural antioxidants in tree nuts such as hydrolysable tannins, flavanoids, and phenolic acids. They hypothesized that AF biosynthesis is stimulated by oxidative stress on the fungus and that compounds that act as antioxidants such as tannins and caffeic acid can suppress AF production.

One of the most in-depth areas of study on the molecular genetics of fungal responses to plant metabolites are those that have looked at a group of metabolites known collectively as oxylipins. Oxylipins are hormone-like molecules that have been implicated as signaling molecules for cross-kingdom communication in plant-pathogen interactions (91). In plants, linoleic (18:2) and linolenic acid (18:3) can be converted by the action of LOX enzymes to $13S$-hydroperoxy linoleic acid ($13S$-HPODE) and $9S$-hydroperoxy linoleic acid ($9S$-HPODE) and $13S$-hydroperoxy linolenic acid ($13S$-HPOTE) and $9S$-hydroperoxy linolenic acid ($9S$-HPOTE) respectively. These plant oxylipins closely resemble the fatty acid-derived compounds known as psi factors produced by aspergilli that can also influence fungal development and toxin production (92). Both *A. flavus* and *A. nidulans* development is affected by the presence of $13S$-HPODE and $9S$-HPODE in a dose-dependent manner (93). Incorporation of pure $13S$-HPODE to cultures of either *A. parasiticus* or *A. nidulans* was shown to repress AF and ST production respectively while pure $9S$-HPODE increased toxin production (94). Interestingly, it appears that growth of the fungus on natural substrates such as maize or peanut seed results in altered expression of seed LOX genes thus leading to changes in the levels of plant oxylipins (95, 96). Postulating that plant oxylipins mimic or interfere with biological activities of endogenous fungal oxylipins, Brodhagen et al. (97) looked at the ability of a maize oxylipin biosynthetic gene (ZmLOX3) to substitute functionally for *A. nidulans* ppo genes that encode dioxygenases involved in synthesis of fungal psi factors. The maize ZmLOX3 gene was introduced into wild-type *A. nidulans* and a ΔppoAC strain (reduced in production of oxylipins psiBa and psiBβ, conidia, and ST) and they observed increased production of conidia and ST in both strains. They also observed that peanut seed pnlox2-3 expression was decreased upon infection by *A. nidulans* Appo mutants compared to levels expressed upon infection by a wild-type strain. These two experiments suggest that oxylipin cross-talk in the host seed-*Aspergillus* interaction may be reciprocal. Though not reported for *A. flavus*, research has shown that maize mutants lacking function of ZmLOX3 had decreased levels of susceptibility to several fungal pathogens including the fumonisin B1 producer, *Fusarium verticillioides* (98). These results support the hypothesis that a specific plant 9-LOX isoform mediates susceptibility of maize to fungal pathogens.
**Genomics**

*Aspergillus flavus* EST and whole genome libraries are invaluable as tools for the identification of genes involved in AF biosynthesis and morphogenesis, as well as fungal pathogenesis/virulence and comparative genomics. Information gleaned from analysis of genome databases and microarray experiments will provide a better understanding of the mechanisms governing AF production and morphogenesis as well as the *A. flavus*-host plant interaction. A number of *Aspergillus* genomes have been sequenced, including *A. flavus* (99), *A. oryzae* (100), *A. nidulans* (101), and *A. niger* (102). Both *A. flavus* EST and two whole genome libraries have been generated and microarrays developed for gene profiling experiments (103, 104). Numerous studies have been performed using EST microarrays for profiling AF and developmental genes in *A. flavus* (37, 105-107). Our lab used EST microarray technology to identify genes differentially expressed in *A. flavus* wild-type veA and veA mutant strains (37). Microarray analysis identified 136 genes that were differentially expressed between the two strains including 27 genes that demonstrated a significant difference in expression both between strains and over time. Of the 136 genes we were able to identify subgroups of genes that exhibited expression profiles similar to those expected for genes involved in AF biosynthesis or sclerotial formation. Guo et al. (108) utilized EST libraries generated from developing peanut (*Arachis hypogaea* L.) seeds at three reproduction stages from a resistant (resistant to *Aspergillus* infection with reduced AF contamination) and a susceptible (susceptible to *Aspergillus* infection with high AF contamination) peanut genotype challenged by *A. parasiticus* and drought stress in the field. A number of resistance-related genes with significant up-regulation were identified from the two libraries.

*Aspergillus oryzae* is a close relative of *Aspergillus flavus* yet it is not a plant pathogen nor does it produce AF. Yu et al. (103) have begun a genome-wide comparison between *A. flavus* and *A. oryzae* in hopes of identifying *A. flavus* genes that are involved in pathogenesis and AF biosynthesis. Preliminary studies indicate that over 95% of the annotated genes are shared between these two fungal species with fewer than 300 genes being unique to each species. In addition to 11,823 *A. flavus* genes, the *A. flavus* whole genome microarrays contain the *A. oryzae* unique genes in addition to genes of interest from maize, *Fusarium* spp., mouse and human genomes. We have performed an experiment using the TIGR *A. flavus* whole genome microarrays to look at differential expression of genes between the wild-type and mutant veA strains (Cary and Calvo, unpublished results). Results identified a number of genes that demonstrated a significant difference in expression both between strains and over time. Of interest was the differential expression of a number of genes involved in nitrogen metabolism, in particular the nitrate reductase (*niaD*) gene. Microarray analysis showed that *niaD* expression was significantly lower in the veA mutant compared to the wild-type. Based on these results an *A. flavus* strain harboring a wild-type or mutant copy of *niaD* were examined for AF production and conidial and sclerotial formation following growth for 5 days on yeast extract-glucose (YGT) agar plates (Fig. 3). Microscopic examination of sclerotia showed that the *niaD* mutant produced fewer and smaller sclerotia than the wild-type strain (Fig. 3A.
and B). Analysis of conidial formation and AF production showed that the niaD mutant produced about 10-fold more conidia than the wild-type but less AF (Fig. 3C). These results indicate that nitrogen metabolism plays a role in both secondary metabolism and development in A. flavus and that expression of some of the genes involved are veA-dependent.

![Micrographs of A. flavus WT and niaD- sclerotia after 5-day growth on YGT agar.](image)

**Concluding Remarks and Future Prospects**

The advent of technologies for the rapid sequencing and annotation of fungal genomes and screening of microarray libraries has provided researchers with additional tools to identify genes involved in the biosynthesis of AF and development in A. flavus as well as its ability to invade plants tissues. The use of comparative genomics between A. flavus and A. oryzae, a close relative of A. flavus that is not a plant pathogen and does not produce AF, should be useful in identifying genes specifically involved in AF production and virulence. Elucidation of the role of plant metabolites and environmental factors such as oxylipins and oxidative stress in AF production and fungal development has opened up new avenues of research on the signaling pathways present in both
the fungus and the host plant and how they may ultimately provide new clues into the ability of the fungus to invade crops, produce AF and survive in the field.

References


102. *Aspergillus niger* v1.0 http://genome.jgi-psf.org/Aspni1/Aspni1.home.html


